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Interactions of β-carotene and cigarette smoke in human bronchial epithelial cells

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Results from recent intervention trials indicated that supplemental β-carotene enhances lung cancer incidence and mortality among smokers. It was hypothesized that β-carotene was exerting its deleterious effects through a prooxidant effect in the smoke-exposed lung. To test this hypothesis we examined the interactions of β-carotene and cigarette smoke in transformed human bronchial epithelial cells. We studied the effects of β-carotene supplementation on rates of gas phase smoke-induced lipid peroxidation, membrane damage and depletion of endogenous antioxidants in BEAS-2B cells. Gas phase cigarette smoke caused cellular β-carotene levels to decrease over time. The oxidation of β-carotene by smoke generated various oxidation products, including 4-nitro-β-carotene, β-apocarotenals and β-carotene epoxides. Peroxidation of membrane lipids by gas phase smoke progressed at a slower rate than did oxidation of β-carotene and incorporation of β-carotene into the cells did not enhance the overall rate of lipid peroxidation. Additionally, lactate dehydrogenase release during smoke exposure was also unaffected by the presence or absence of β-carotene in cells. β-Carotene incorporation in cells was not found to accelerate the rates of α-tocopherol and glutathione depletion by cigarette smoke. Our results indicate that β-carotene is more sensitive than lipids to cigarette smoke oxidation, but that this preferential oxidation of β-carotene does not lead to a prooxidant effect in human bronchial epithelial cells.

Introduction

On the basis of epidemiological evidence, dietary β-carotene has been postulated to be an effective agent for prevention of lung cancer (1–3). Surprisingly, results from two randomized trials, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) trial in Finland and the β-Carotene and Retinol Efficacy Trial (CARET) in the USA showed that supplemental β-carotene increased the risk of lung cancer in high risk groups of smokers (4,5). These unexpected results led to a re-examination of the antioxidant hypothesis of β-carotene in the smoke-exposed lung. The confusion regarding carotenoid anticarcinogenic efficacy may be attributed in part to a poor understanding of the interactions between cigarette smoke components, β-carotene and lung epithelial cells.

Cigarette smoke contains high concentrations of two distinctly different populations of free radicals, one in the tar component and the other in gas phase smoke. The tar component is defined as the material that is trapped by a Cambridge filter that retains 99.99% of the particles >0.1 μm, whereas gas phase smoke is the material that passes through the Cambridge filter. Tar consists primarily of an amorphous mixture of polycyclic hydrocarbons, polyphenols and quinones and is strongly reducing in its overall redox character (6,7). Gas phase smoke predominantly contains steady-state concentrations of reactive carbon and oxygen-centered radicals with lifetimes of <1 s (8). Cigarette smoke thus poses a mixed oxidative challenge to the cells. While the strong oxidants in gas phase smoke can rapidly initiate lipid, DNA and protein oxidation, the polyphenol–quinone redox couples in tar can more slowly generate radicals over a sustained period.

Carotenoids display antioxidant properties (9) and are generally thought to prevent oxidative damage, as would be caused by cigarette smoke. The leading hypothesis for this anticarcinogenic effect of carotenoids is that they act as antioxidants, trapping free radicals and other reactive oxidants in cigarette smoke. However, β-carotene antioxidant chemistry is known to display a striking dependence on oxygen tension (p_{O2}) (10). Burton and Ingold (10) reported that at the low p_{O_2} typical of most tissues (<50 torr) β-carotene acts as a chain-breaking antioxidant, whereas at higher p_{O_2} β-carotene is readily autoxidized and may display prooxidant behavior. This is believed to result from the ability of oxygen to reversibly add to β-carotene-derived radicals, thereby generating reactive peroxyl radicals, which then propagate radical chains (equations 1 and 2):

$$\beta\text{-carotene} + ROO^{\bullet} \rightarrow ROO\text{–}\beta\text{-carotene}^{\bullet} \quad (1)$$

$$ROO\text{–}\beta\text{-carotene} + O_2 \rightarrow ROO\text{–}\beta\text{-carotene–}OO^{\bullet} \quad (2)$$

These β-carotene peroxyl radicals generated could facilitate β-carotene autoxidation and react with other molecules to produce prooxidant effects.

An attractive hypothesis that was put forth to explain the results of the CARET and ATBC trials was that β-carotene may be acting as a prooxidant in the lungs of smokers (11). The basis of this hypothesis was that smoke-borne oxidants, including peroxyl radicals and nitrogen oxides, could initiate β-carotene autoxidation at the relatively high oxygen tension in the lung (150 torr). This could lead to a secondary oxidation of lung biomolecules by β-carotene and smoke-derived radical intermediates. The resulting prooxidant interaction of β-carotene with smoke could enhance oxidative injury beyond that caused by smoke alone.

A previous study from our laboratory examined the interactions of β-carotene and cigarette smoke in model systems (12). This study identified 4-nitro-β-carotene as a unique product of the reactions between β-carotene and cigarette

Abbreviations: AAPH, 2,2′-azo-bis(2-amidinopropane)dihydrochloride; BHT, butylated hydroxytoluene; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; GC/MS, gas chromatographic–mass spectrometric; KGM, keratinocyte growth medium; LDH, lactate dehydrogenase; MeLin, methyl linoleate; PBS, phosphate-buffered saline; THF, tetrahydrofuran.

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smoke. It also demonstrated that β-carotene failed to produce a prooxidant interaction with cigarette smoke in liposomal model systems. The aim of the present study was to extend the findings of the previous research to living cells. Here we have examined the possible prooxidant or antioxidant actions of β-carotene in immortalized human bronchial epithelial cells exposed to cigarette smoke.

Materials and methods

Chemicals

Immortalized human bronchial epithelial cells (BEAS-2B) were obtained from the American Type Culture Collection (Rockville, MD). All-*trans*-β-carotene was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). *d*-α-Tocopherol was a gift from Henkel Fine Chemicals (LaGrange, IL). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was from Avanti Polar Lipids (Alabaster, AL). *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane were from Pierce (Rockford, IL). Research grade cigarettes (1R3) were provided by the University of Kentucky Tobacco and Health Research Institute (Lexington, KY). Cambridge filter pads were from Performance Systematics (Caledona, MI). All other chemicals were of the highest purity available.

Cell culture

BEAS-2B cells were cultured using serum-free, hormone-supplemented medium (keratinocyte growth medium, KGM; Clonetics, San Diego, CA) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown on plastic flasks, removed by brief trypsinization, and 250 000 cells were plated on 25 mm diameter polyester filter supports with a 3 μm pore size (Transwell-Clear; Corning Costar, Cambridge, MA), which were inserted into 6-well culture dishes.

Delivery of β-carotene to cells

Two different methods of β-carotene delivery to BEAS-2B cells were investigated. Cells were exposed to β-carotene solubilized in tetrahydrofuran (THF) and ethanol. Cells were also exposed to β-carotene incorporated into DPPC liposomes. For the solvent delivery method β-carotene stock solubilized in THF and ethanol (1:1 v/v) was added to the KGM with rapid vortex mixing to achieve final β-carotene concentrations of either 0.62, 1.25 or 2.5 μM in the medium. THF was passed through alumina to remove peroxides prior to use. The amount of THF or ethanol in the medium never exceeded 0.2% by volume. In the liposome delivery method appropriate volumes of β-carotene stock in hexane and DPPC dissolved in chloroform were evaporated under nitrogen and resuspended in THF and ethanol (0.1% each). Liposomes were produced by injecting the lipid and β-carotene suspension into a flask containing KGM with rapid vortex mixing for final concentrations of 500 μM DPPC and 0.62, 1.25 or 2.5 μM β-carotene. In both modes of delivery 4 ml of β-carotene-supplemented medium was added to the basolateral (2.5 ml) and apical (1.5 ml) chambers of each well containing BEAS-2B cells for final concentrations of 2.5, 5 or 10 nmol β-carotene/well. The cells were exposed to β-carotene-supplemented culture medium for 24 h prior to cigarette smoke exposure studies. Control cells received equivalent amounts of DPPC liposomes or THF and ethanol without β-carotene.

Cigarette smoke exposure

For smoke exposure experiments cell medium was aspirated from the Transwells and the cells were rinsed with phosphate-buffered saline (PBS), pH 7.2. Two milliliters of PBS was added to the basolateral site of each well. To facilitate interaction of gas phase smoke with BEAS-2B cells no fluid was added to the apical compartments. The Transwells were placed in glass chambers with separate inlets for compressed air and cigarette smoke. Cells were exposed to 1200 ml of gas phase smoke every 10 min using a smoking apparatus consisting of a 60 ml syringe, a three-way stopcock and an inline filter. Smoke from research grade 1R3 cigarettes was passed through a Cambridge glassfiber filter to remove the tar fraction prior to introduction of smoke into the chamber. The chamber was purged with fresh compressed air prior to each smoke exposure. These experiments were conducted using an oxygen tension of 150 torr for the cigarette smoke and air mixture to approximate the conditions to which the lung epithelium would be exposed *in vivo*.

Analysis of β-carotene depletion

After treatment the cells were rinsed with PBS and subsequently lysed with 10 μmol SDS. Butylated hydroxytoluene (BHT, 100 nmol, 22 μg) was added to the cell suspension as an antioxidant to prevent adventitious oxidation of β-carotene. β-Carotene was extracted from cells using sequential extractions with hexane:ethanol (1:1 v/v), hexane:ethanol (2:1 v/v) and hexane. The hexane extracts were pooled and evaporated *in vacuo*. α-Tocopherol propionate was added as an injection standard, the samples were redissolved in mobile phase and analyzed by reverse phase HPLC with diode array detection (Hewlett Packard, Palo Alto, CA). A Spherisorb ODS-2 column (5 μM, 250×10 mm; Alltech Associates, Deerfield, IL) eluted with methanol:hexane (85:15 v/v) at 1.5 ml/min was used for analysis. β-Carotene was detected at 450 nm and α-tocopherol propionate at 286 nm.

Lactate dehydrogenase (LDH) release

LDH release from the cells was quantified using a LD-L 10 kit (Sigma, St Louis, MO). Total cellular LDH was quantified from cell lysates sonicated in the presence of 1% Triton X-100.

Lipid peroxidation analysis

BEAS-2B cells were lysed in SDS (10 μmol) and membrane lipids were extracted three times with chloroform:methanol (2:1 v/v) containing BHT (100 nmol). The pooled chloroform fractions were evaporated under nitrogen and redissolved in methanol (1.5 ml). The samples were reduced with $NaBH_4$ (5 mg) for 30 min at room temperature, transesterified with KOH/methanol (100 mg in 1 ml) and derivatized to their trimethylsilyl ethers using 100 μl of BSTFA + 1% trimethylchlorosilane at 65°C for 45 min. After catalytic hydrogenation using platinum oxide (3 mg) and H_2 for 2 min the peroxidation of membrane lipids was assessed by measuring levels of 9'-OH methyl linoleate (MeLin) using the isotope dilution gas chromatographic–mass spectrometric (GC/MS) assay of Stratton and Liebler (13). GC/MS analyses were done with a Fisons MD800 mass spectrometer coupled to a Carlo Erba 5000 series GC (Fisons Instruments, Beverly, MA).

Glutathione depletion

For determination of glutathione content the cells were harvested in 10% perchloric acid containing 1 mM bathophenanthrolinedisulfonic acid. Glutathione content of cells was determined using the procedure of Fariss and Reed (14). The procedure involved initial formation of *S*-carboxymethyl derivatives of free thiols followed by conversion of free amino groups to 2,4-dinitrophenyl derivatives and separation by ion exchange HPLC using γ-glutamyl glutamate as an internal standard.

α-Tocopherol depletion

For α-tocopherol depletion experiments KGM cell medium was supplemented with 1.25 μM α-tocopherol dissolved in 0.2% ethanol for 24 h prior to smoke exposure. For α-tocopherol analyses cells were sonicated in a mixture of ethanol (2 ml), α-tocopherol-d_6 (internal standard, 10 nmol) and SDS (10 μmol). The samples were extracted with hexane and the hexane extracts were evaporated under N_2. The samples were converted to their trimethylsilyl ethers with 100 μl of BSTFA containing 10% trimethylchlorosilane and dimethylformamide (100 μl) at room temperature for 2 h. The α-tocopherol content of cells was assessed by GC/MS assay using selected ion monitoring as described previously (15).

Identification of β-carotene smoke oxidation products

β-Carotene and its smoke oxidation products were analyzed by liquid chromatography–mass spectrometry using an Allsphere ODS-2 5 μm HPLC column (4.6×250 mm) (Alltech Associates) with gradient elution at 1 ml/min (16). The mobile phase initially consisted of 100% solvent A (acetonitrile/methanol/ammonium acetate, 85/15/0.1 v/v/w). At 8 min solvent B (2-propanol) was introduced as a linear gradient to 30% by 12 min. The conditions were maintained until 25 min. From 25 to 30 min the mobile phase was returned to 100% solvent A using a linear gradient. β-Carotene oxidation products were detected by monitoring the UV/vis and MS spectra. MS analyses were performed on a Finnigan TSQ-7000 triple quadrupole mass spectrometer using an atmospheric pressure chemical ionization source (Finnigan MAT, San Jose, CA). Mass spectra were obtained in negative ion mode.

Statistical analysis

Results are expressed as means ± SEM. Statistical significance within sets of data was determined by one way analysis of variance (ANOVA) followed by individual comparisons using Bonferroni's correction for factorial analysis of variance.

Results

Cellular uptake of β-carotene

Since β-carotene is a highly lipophilic compound poorly taken up by cells in culture, preliminary experiments were conducted to optimize its uptake by BEAS-2B cells. Despite published reports (17,18), addition of β-carotene in ethanol to culture medium was not found to be a good delivery method due to the limited solubility of β-carotene in ethanol (data not shown).

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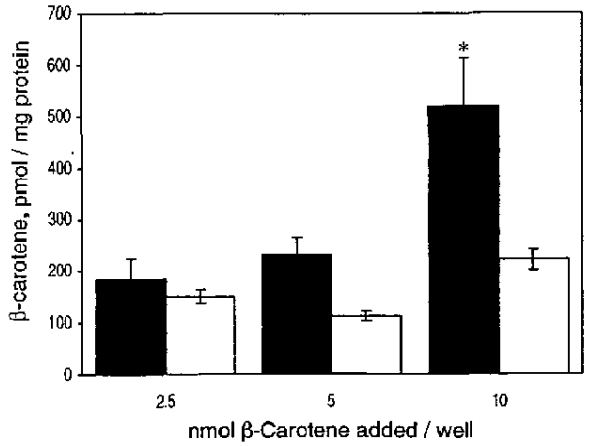

Fig. 1. Uptake of β-carotene by BEAS-2B cells exposed to extracellular β-carotene solubilized in either THF and ethanol (■) or incorporated into DPPC liposomes (□) (*$P < 0.05$, compared with the respective 2.5 nmol β-carotene-exposed cells).

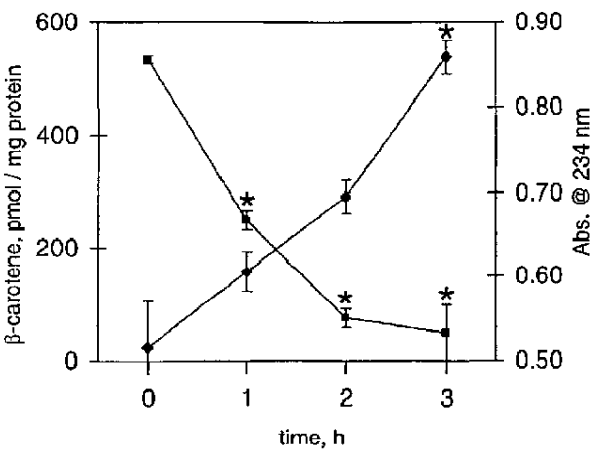

Fig. 2. β-Carotene depletion and increases in lipid peroxidation level in BEAS-2B cells exposed to 50 mM AAPH (*$P < 0.05$, compared with the corresponding 0 h values).

We investigated the uptake of β-carotene by: (i) exposure of cells to β-carotene solubilized in THF and ethanol; (ii) exposure of cells to β-carotene incorporated into DPPC liposomes.

Addition of β-carotene solubilized in THF and ethanol was consistently found to result in higher uptake by BEAS-2B cells as compared with β-carotene incorporated into liposomes for delivery into the cells (Figure 1). To ensure that the solvent delivery method incorporated β-carotene into cells in a physiologically relevant manner the cells were oxidized with 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and depletion of β-carotene and increases in levels of lipid peroxidation were monitored over time (Figure 2). β-Carotene levels in cells decreased over time, suggesting that β-carotene was associated with the cells in a physiologically relevant manner and could participate in the oxidation chemistry of the cells. All subsequent experiments were performed by delivering 5 nmol β-carotene in 4 ml of medium/well (equivalent to a final extracellular concentration of 1.25 μM β-carotene) dissolved in 0.1% each THF and ethanol for 24 h prior to smoke exposures.

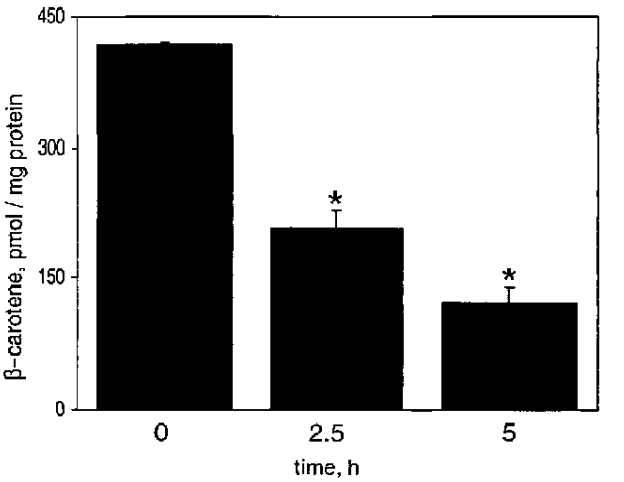

Fig. 3. Time course of β-carotene depletion in BEAS-2B cells exposed to gas phase cigarette smoke (*$P < 0.05$, compared with 0 h).

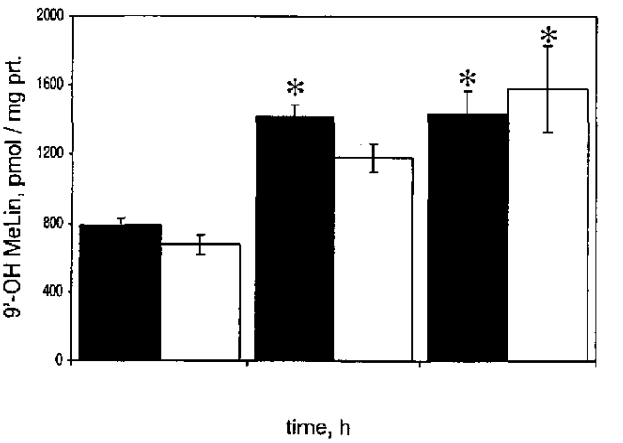

Fig. 4. Lipid peroxidation in BEAS-2B cells supplemented with vehicle (■) or 5 nmol β-carotene (□) and exposed to gas phase smoke. Formation of 9'-OH MeLin was measured as described in Materials and methods (*$P < 0.05$, compared with the respective 0 h smoke-exposed cells).

Depletion of β-carotene levels in BEAS-2B cells by gas phase cigarette smoke

Prior to testing our hypothesis that smoke-driven β-carotene autoxidation exerted prooxidant effects it was necessary to establish that gas phase smoke was capable of oxidizing cellular β-carotene. BEAS-2B cells supplemented with an extracellular concentration of 1.25 μM β-carotene for 24 h were exposed to gas phase smoke for 2.5 and 5 h and depletion of β-carotene over time was measured. Gas phase smoke caused significant depletion of β-carotene levels in BEAS-2B cells, with ~75% of β-carotene oxidized by the end of the 5 h smoke exposure (Figure 3).

Oxidation of cellular lipids by gas phase cigarette smoke

To determine whether smoke-driven autoxidation of β-carotene resulted in prooxidant effects in BEAS-2B cells we investigated the effects of β-carotene on gas phase smoke-induced lipid peroxidation of membrane lipids. Gas phase smoke exposure significantly enhanced lipid peroxidation in BEAS-2B cells, as assessed by formation of 9'-OH MeLin (Figure 4). However,

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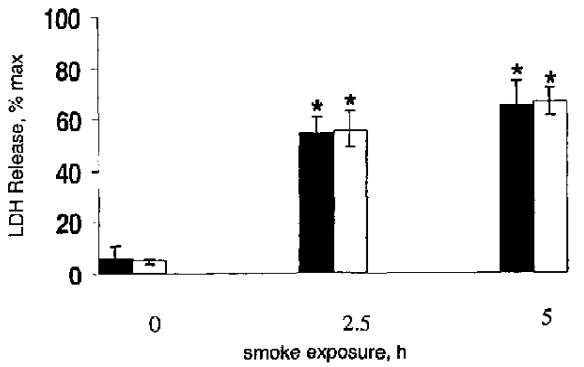

Fig. 5. LDH release from BEAS-2B cells supplemented with vehicle (■) or 5 nmol β-carotene (□) and exposed to gas phase smoke. (*$P < 0.05$, compared with 0 h smoke-exposed cells).

supplementation of the cells with β-carotene did not cause an enhancement of smoke-induced lipid peroxidation. Overall, β-carotene supplementation led to a slight attenuation of lipid peroxidation levels in BEAS-2B cells at 2.5 h, but this sparing effect of β-carotene was lost by the end of the 5 h smoke exposure.

LDH release from BEAS-2B cells exposed to gas phase smoke

Membrane damage to BEAS-2B cells by exposure to gas phase smoke was assessed by measuring the release of the cytoplasmic enzyme LDH into the medium. Exposure of cells to gas phase smoke resulted in a significant loss of membrane integrity, as indicated by the amount of LDH released by the cells (Figure 5). By the end of the 5 h cigarette smoke exposure ~60% of the LDH from the cells had been released into the medium. β-Carotene supplementation did not affect viability of the cells. Moreover, supplementation of cells with β-carotene did not affect the extent of membrane damage caused by gas phase smoke.

Antioxidant depletion in BEAS-2B cells by gas phase cigarette smoke

We then examined the possibility that smoke-driven β-carotene autoxidation was accelerating the rates of depletion of endogenous cellular antioxidants. Water-soluble glutathione and lipid-soluble α-tocopherol were the representative antioxidants studied. In the case of glutathione gas phase smoke caused rapid depletion of endogenous glutathione levels in BEAS-2B cells (Figure 6). More than 90% of cellular glutathione was oxidized by the end of the 30 min gas phase smoke exposure. Supplementation of the cells with β-carotene did not enhance this rate of smoke-driven glutathione depletion.

Since the cells were grown in serum-free medium, their endogenous levels of α-tocopherol were very low. Hence, we supplemented the cells with 1.25 μM α-tocopherol for 24 h prior to the smoke exposure studies. In contrast to glutathione, cellular α-tocopherol was much more resistant to gas phase smoke autoxidation (Figure 7). No statistically significant depletion of cellular α-tocopherol levels was observed during the 5 h smoke exposure, even though there was a general trend towards decreased levels. Although the levels of α-tocopherol appear slightly lower in β-carotene-supplemented cells, the differences were not statistically significant.

Characterization of β-carotene oxidation products

The β-carotene oxidation products formed in cells by exposure to gas phase smoke were characterized on the basis of their

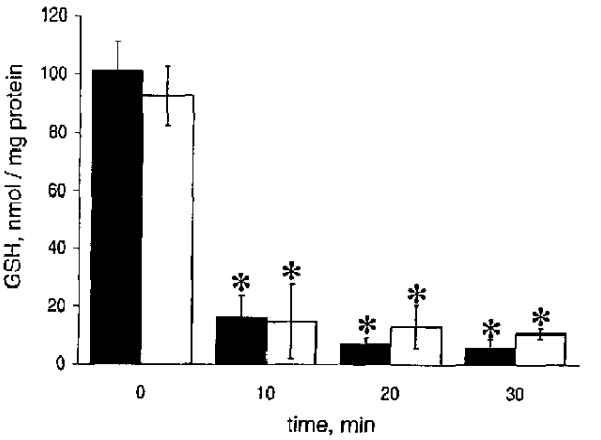

Fig. 6. Depletion of glutathione (GSH) in BEAS-2B cells supplemented with vehicle (■) or 5 nmol β-carotene (□) and exposed to gas phase smoke (*$P < 0.05$, compared with smoke-exposed cells).

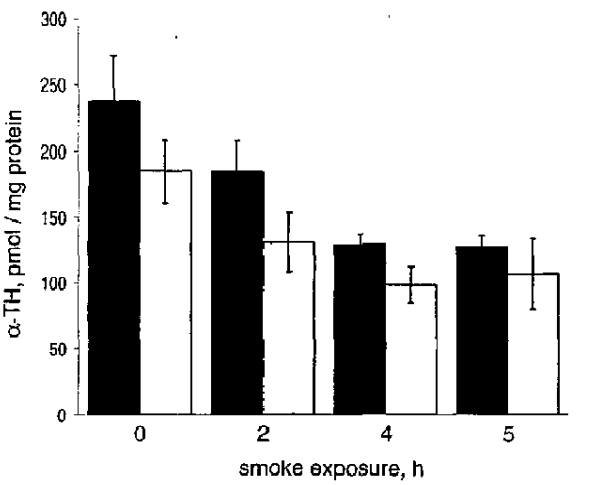

Fig. 7. Depletion of α-tocopherol in BEAS-2B cells supplemented with vehicle (■) or 5 nmol β-carotene (□) and exposed to gas phase smoke (*$P < 0.05$, compared with smoke-exposed cells).

UV/vis spectra, mass spectra and HPLC retention times. The principal oxidation products were identified as β-apo-14'-apocarotenal, β-apo-12'-apocarotenal, β-apo-10'-apocarotenal, 4-nitro-β-carotene, 5,8-epoxy-β-carotene and 5,6-epoxy-β-carotene (Table I). The HPLC and spectral data for these compounds corresponded to previously reported values (12,16,19,20). β-Carotene smoke oxidation products formed in BEAS-2B cells were also observed in an earlier study done in our laboratory with smoke oxidation of β-carotene in a liposomal model system (12). Of these smoke oxidation products 4-nitro-β-carotene is a compound that has been shown to be a unique marker for cigarette smoke oxidation of β-carotene. The other β-carotene oxidation products observed here, the β-apo-carotenals and β-carotene epoxides, have been observed in previous studies of the antioxidant reactions of β-carotene during lipid oxidation (12,13,21,22).

Discussion

The mechanism(s) by which β-carotene resulted in increased lung cancer mortality among smokers in recent intervention

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Table I. UV/Vis, HPLC and MS data for β-carotene and its major oxidation products formed during gas phase smoke exposure of BEAS-2B cells

Peak	Compound	Retention time (min)	UV/vis λ_{max}[a]	Negative APCI (*m/z*)
1	β-Apo-14'-apocarotenal	6.9	**398**	310
2	β-Apo-12'-apocarotenal	9.4	**422**	350
3	β-Apo-10'-apocarotenal	10.3	**442**	376
4	4-Nitro-β-carotene	16.7	**454**	580[c]
5	5,8-Epoxy-β-carotene	18.2	**428**, 452	552b
6	5,6-Epoxy-β-carotene	19.3	**446**, 474	552[b]
8	β-Carotene	24.8	**452**, 476	536[b]

[a]The most intense absorbance bands are listed in bold.
[b]$(M^{\bullet})^{-}$.
[c]$(M-H)^{-}$.

trials has yet to be defined. One proposed explanation for these deleterious effects of β-carotene was that it acts as a prooxidant in smoke-exposed lungs and exacerbates oxidative damage caused by cigarette smoke. Here, our work with human bronchial epithelial cells demonstrates that gas phase smoke caused oxidation of membrane lipids, cellular β-carotene and other endogenous antioxidants. Gas phase smoke oxidation of β-carotene in cells generated 4-nitro-β-carotene, β-carotene epoxides and β-apo-carotenals as oxidation products. These products are similar to those observed earlier with gas phase oxidation of β-carotene in a homogeneous solution system (12). However, this oxidation of β-carotene by gas phase smoke did not translate into a direct prooxidant effect in human bronchial epithelial cells.

Our goal in these studies was to examine the interaction of β-carotene, smoke and cellular constituents in the most physiologically relevant yet practically accessible *in vitro* model. Our choice of BEAS-2B cells as the cell culture model is based on the successful use of these cells in similar previous studies with both solution phase and airborne toxicants (23–27). These transformed normal human bronchial epithelial cells have reduced levels of endogenous antioxidants compared with primary normal bronchial cells (28). This allowed us to manipulate antioxidant capacity of the cells and study effects of specific antioxidants added to the system. Similarly, use of the Transwell system allowed direct exposure of the cells to gas phase smoke components (23,24,27). The smoke exposures were done with air–smoke mixtures that would approximate both the smoke burden and oxygen tension to which the lung epithelium would be exposed *in vivo*. A final key consideration in this study is what level of β-carotene supplementation best reflects β-carotene levels in lung tissue of human subjects taking β-carotene supplements. Redlich *et al.* reported that β-carotene levels in cells obtained by bronchoalveolar lavage from 12 CARET participants had levels of 46.3 ± 53.3 pmol/10^6cells, compared with 4.5 ± 4.8 pmol/10^6 cells prior to supplementation (29). Our supplementation with 5 nmol β-carotene per Transwell resulted in cellular β-carotene levels of ~400 pmol/mg protein, or ~50 pmol/10^6 cells. Thus, our experimental model reasonably approximated the conditions of β-carotene and smoke interactions in individuals taking β-carotene supplements *in vivo*.

Another challenge in this study was to supplement bronchial epithelial cells with β-carotene in a physiologically relevant manner. Previous studies have added β-carotene dissolved in ethanol to the cell culture medium (17,18,30). β-Carotene has very limited solubility in ethanol and probably was present as a fine suspension of β-carotene aggregates at the high concentrations used in those studies. We found that the most effective way of supplementing BEAS-2B cells with β-carotene was to use a mixture of THF:ethanol (1/1 v/v) for solubilizing β-carotene. This is similar to the method previously reported by Bertram *et al.* (31). Oxidation with the water-soluble azo initiator AAPH demonstrated that β-carotene added to the cells using THF and ethanol was available to react with smoke-borne oxidants in the cells and hence was incorporated into the cells in a physiologically relevant manner.

An important test of the model was the extent to which smoke induced β-carotene oxidation in BEAS-2B cells. The results indicated that gas phase smoke exposure caused extensive oxidation of β-carotene. The products formed are those identified previously in various β-carotene oxidation systems, including smoke oxidation. An important observation in this context is the formation of 4-nitro-β-carotene, which is generated by nitrogen oxides in smoke (12). The identification of 4-nitro-β-carotene clearly establishes that smoke directly oxidized β-carotene in this model.

We hypothesized that a β-carotene prooxidant effect in smoke-exposed cells could be manifested in two different ways. First, radical intermediates in β-carotene oxidation could initiate radical oxidation of other cellular molecules, including membrane lipids. Thus, we analyzed the effects of β-carotene supplementation on smoke-induced lipid peroxidation and LDH release. Second, radical intermediates in β-carotene oxidation could oxidize other cellular antioxidant molecules and compromise cellular defense against smoke oxidants. Our results indicated that β-carotene did not enhance membrane lipid peroxidation or LDH leakage. Further, β-carotene did not affect the kinetics of depletion of either glutathione or α-tocopherol by smoke.

These results, taken together with those of our previous studies in a liposome model (12), indicate that prooxidant effects of β-carotene are unlikely to contribute to increased incidence of cancer in smokers taking β-carotene supplements. Although it is still possible that localized prooxidant chemistry of β-carotene selectively oxidizes certain critical targets, our methods could not detect such changes. Nevertheless, this scenario seems unlikely. Other mechanisms by which β-carotene–smoke interactions enhance lung carcinogenesis are worthy of further exploration. Wang *et al.* recently studied this interaction in ferrets supplemented with β-carotene and exposed to cigarette smoke *in vivo* (32). Ferrets absorb β-carotene extensively, as do humans, and are considered useful models for carotenoid actions *in vivo*. Extensive conversion of β-carotene to oxidation products was observed in subcellular fractions from smoke-exposed ferrets. β-Carotene also

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enhanced smoke-induced epithelial cell proliferation and squamous metaplasia. Moreover, carotenoid supplementation also enhanced smoke repression of RARβ expression and enhanced induction of c-Fos and c-Jun. These observations suggest that β-carotene oxidation products may exert effects on retinoid signaling and affect the status of other nuclear transcription factors as well. Further exploration of this hypothesis could provide a fresh perspective on the deleterious interaction of β-carotene and smoke.

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